Exhibit E

ESTABLISHMENT OF A TUMOR-SPECIFIC IMMUNOTHERAPY MODEL UTILIZING TNP-REACTIVE HELPER T CELL ACTIVITY AND ITS APPLICATION TO THE AUTOCHTHONOUS TUMOR SYSTEM[1]

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Preinduction of potent hapten-reactive helper T cell activity and subsequent immunization with hapten-coupled syngeneic tumor cells result in enhanced induction of tumor-specific immunity through T-T cell collaboration between anti-hapten helper T cells and tumor-specific effector T cells. On the basis of this augmenting mechanism, a tumor-specific immunotherapy protocol was established in which a growing tumor regresses by utilizing a potent trinitrophenyl (TNP)-helper T cell activity. C3H/He mice were allowed to generate the amplified (more potent) TNP-helper T cell activity by skin painting with trinitrochlorobenzene (TNCB) after pretreatment with cyclophosphamide. Five weeks later, the mice were inoculated intradermally with antigenic transplantable X5563 tumor cells. When TNCB was injected into X5563 tumor mass, an appreciable number of growing tumors, in the only group of C3H/He mice in which the amplified TNP-helper T cell activity had been generated were observed to regress (regressor mice). These regressor mice were shown to have acquired tumor-specific T cell-mediated immunity. Such immunity was more potent than that acquired in mice whose tumor was simply removed by surgical resection. These results indicate that in situ TNP haptenation of the tumor cells in TNP-primed mice can induce the enhanced tumor-specific immunity leading to the regression of a growing tumor. Most importantly, the present study further investigates the applicability of this TNP immunotherapy protocol to an autochthonous tumor system. The results demonstrate that an appreciable percent of growing methylcholanthrene-induced autochthonous tumors regressed by the above TNP immunotherapy protocol. Thus, the present model provides an effective maneuver for tumor-specific immunotherapy in syngeneic transplantable as well as autochthonous tumor systems.

On the basis of the hypothesis of Mitchison (1) concerning manipulations that might augment tumor-specific immunity, numerous attempts to enhance the im-

munogenicity of tumor-associated transplantation antigens (TATA)[3] by coupling additional antigenic determinants on the tumor cell surface have been reported (2-6). Helper T cells can collaborate with effector T cell precursors, such as cytotoxic cell precursors, to enhance immune responses against various antigens including TATA (7). If additional determinants coupled onto the tumor cell act as helper determinants. it is therefore conceivable that preinduction of helper T cell activity to these additional determinants could induce much higher anti-TATA immune responses at the time of stimulation of tumor cells conjugated with the corresponding antigenic determinants.

We defined conditions under which enhanced immune resistance to tumors could be generated by preinducing trinitrophenyl (TNP) hapten-reactive T cells, and by subsequently immunizing with TNP-coupled syngeneic tumor cells (8, 9). This system is designed to induce the most efficient generation of tumor-specific effector T cell activity in vivo by virtue of the close linkage of hapten-reactive helper T cells and TATA-specific effector precursor T cells in the microenvironment at the time of stimulation with hapten-coupled tumor cells. Our previous results demonstrated that the generation of potent TNP-helper cell activity after elimination of suppressor cell activity was a prerequisite for amplified generation of in vivo protective immunity, and a T-T cell interaction mechanism between TNP-helper T cells and anti-TATA effector T cell precursors was thus suggested to be essential to such a phenomenon (10). These results prompted us to establish an immunotherapeutic protocol in tumor-bearing animals in which such potent TNP-helper T cells were used.

In the present study, when TNP was introduced into the tumor mass of tumor-bearing mice in which the amplified TNP-reactive helper T cell activity had been generated, in situ trinitrophenylation of tumor cells resulted in a high incidence of complete regression of growing tumors. We demonstrated that the tumor regression was accompanied by the concurrent generation of a potent tumor-specific T cell immunity, suggesting on the above T-T cell collaboration mechanism was functioning in this tumor immunotherapy protocol. More importantly, the present study also investigates whether such an immunotherapeutic potential realized in the TNP-helper

[3] Abbreviations used in this paper: TATA, tumor-associated transplantation antigens; TNCB, trinitrochlorobenzene; MCA. 3-methylcholanthrene: Cy. cyclophosphamide; CTL. cytotoxic T lymphocyte: i.d.. intradermal; DTH. delayed-type hypersensitivity.

TABLE I

Comparison of acquisition of tumor-specific immunity after TNP immunotherapy and surgical resection of tumor

Group	Mice	Incidence of Resistance Against Tumor Challenge[a]
A	Normal	0/10
B	After regression of tumor by TNP immunothera-py[b]	11/12
C	After surgical resection of tumor[c]	2/10

[a] Mice were challenged i.d. with 10^5 viable X5563 tumor cells, and incidence of resistance was determined 3 weeks after the tumor challenge.

[b] C3H/He mice whose X5563 tumor regressed in the TNP immunotherapy model as shown in Fig. 1 (group E) were used 3 wk after initial tumor implantation.

[c] C3H/He mice were inoculated i.d. with 10^6 X5563 tumor cells and growing tumors were surgically resected 7 days later. Mice were used 2 wk after tumor removal.

growth of metastasized tumor cells. Thus, the difference in incidence of anti-X5563 immunity between two groups above indicates more potent anti-X5563 immune resist-ance was retained in mice whose tumors regressed by virtue of the TNP immunotherapy.

The development of stronger anti-X5563 immune re-sistance in regressor mice was also confirmed by com-paring the tumor-neutralizing activity of spleen cells from these mice to that of mice whose tumors were surgically resected. Winn assay performed with these two groups of spleen cells at a lower spleen to tumor cell ratio that appreciably stronger tumor-neutralizing ac-tivity was generated in the regressor mice by TNP im-munotherapy than in the mice that had tumors resected surgically (Table II).

Additional experiments were performed to test the na-ture and specificity of the effector mechanism acquired by X5563 tumor regressor mice. Winn assays with the use of spleen cells from the regressor mice also demon-strated that these spleen cells resulted in complete neu-tralization of X5563 tumor cells when admixed, but failed to exhibit 1) tumor neutralization against X5563 tumor after the treatment of the spleen cells with anti-Thy-1.2 plus C (Table III), and 2) tumor neutralization against another syngeneic tumor MH134 hepatoma (Table IV). These results indicate the T cell nature and specificity of anti-X5563 immunity acquired by the regressor mice in the TNP immunotherapy model.

Application of the TNP immunotherapeutic protocol to an autochthonous tumor system. In the process of application of the present tumor-specific immunotherapy model to a chemical carcinogen-induced autochthonous

tumor system. we extended this TNP immunotherapy model to another transplantable. chemical carcinogen-induced tumor (MCH-1-A1) system in which the tumor was recently induced in C3H/He mice by MCA and has been maintained in our laboratory (less than 10 passages in vivo). A similar protocol to that performed in the X5563 tumor system was used and the results are illustrated in Figure 2. In this experiment. TNCB injection into the MCH-1-A1 tumor mass from Cy→TNCB-painted mice led to a high incidence of tumor regression. In contrast to the lack of tumor regression when in situ TNP modification was performed in mice not primed to TNP. Thus, this TNP immunotherapy system is also applicable to another recently established transplantable. chemical carcino-gen-induced fibrosarcoma tumor system.

The successful regression of growing tumors in an MCA-induced transplantable tumor system by using the TNP immunotherapy regimen encouraged us to test the applicability of this immunotherapy protocol to an MCA-induced autochthonous tumor system. The primary tu-mor was induced in 500 female C3H/HeN mice at 8 wk of age by injecting 0.5 mg MCA in 0.1 ml olive oil subcu-taneously. Four weeks after the MCA inoculation, one half of the group of mice received the combined treatment of Cy injection and TNCB painting. which was capable of inducing the amplified TNP-reactive helper T cell activity. and the remainder were untreated. The mice began to develop a primary. subcutaneous tumor about 8 wk after the MCA treatment. At 9 wk after the MCA injection. 20 to 30% of mice in both TNP-helper-positive and -negative groups bore a tumor in the range of 6 to 9 mm in diameter. Histological examination of 10 autochthonous' tumors randomly selected (five mice in each group) revealed that all were fibrosarcoma. Mice that did not receive tumor excision were collected and each group was randomly divided into two groups depending on whether mice were treated with the intratumoral injection of 0.15 ml of 1% TNCB. Therefore. the experiment consisted of four groups: group A: MCA injection only; group B: MCA injec-tion→intratumoral TNCB injection: group C: MCA injec-tion→the combined treatment of Cy plus TNCB painting: and group D: MCA injection→the above combined treat-ment for priming of potent TNP-helper T cells→intratu-moral TNCB injection. The tumor growth of four groups of animals is shown in Figure 3. Most tumors in three groups of mice (groups A. B and C). except for only one animal in group B. continued to grow until the animal died. although the growth rate exhibited varied patterns. Importantly. however. an appreciable number (11 of 25)

TABLE II

Comparison of tumor-neutralizing activity between spleen cells from mice after tumor regression after TNP immunotherapy and from mice after surgical resection of tumor

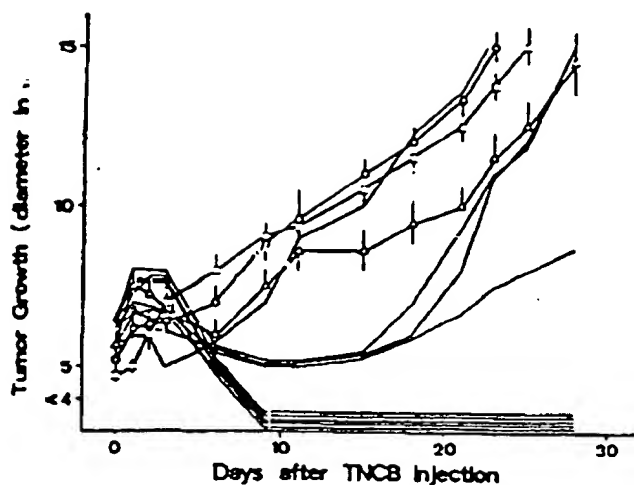| Spleen Cells from Mice	Spleen:Tumor Cell Ratio	Tumor Growth[a] (mm diam)		
		Day 7	Day 10	Day 12
tion of tumor by TNP immunotherapy[b]	100:1	5.8 ± 1.3	9.6 ± 1.0	13.5 ± 0.3
al resection of tumor[c]	100:1	<3.0	<3.0	<3.0
	100:1	<3.0	<3.0	<3.0
After regression of tumor by TNP immunotherapy	10:1	4.8 ± 0.9	9.3 ± 0.9	12.5 ± 1.0
After surgical resection	10:1	<3.0	<3.0	5.0 ± 1.5
of tumor	10:1	<3.0	7.5 ± 0.6	10.3 ± 1.2

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Figure 2. Induction of tumor regression in MCH-1-A1 tumor-bearing mice by using the TNP immunotherapy regimen. C3H/He mice received the combined treatment of Cy injection and TNCB painting. Five weeks after TNCB painting. mice were inoculated i.d. with 10⁶ viable MCH-1-A1 tumor cells. The in situ TNP haptenation identical to that in Fig. 1 was performed 7 days after tumor cell inoculation. Tumor growth was individually scored and expressed by tumor diameter (——). Tumor growth in control groups was expressed by mean diameter ± SE of seven mice per group. (O——O). (Δ——Δ). and (□——□) indicate tumor cell inoculation only. tumor cell inoculation→in situ TNP haptenation, and the above combined treatment for TNP priming→tumor cell inoculation. respectively.

T cell immunity was more potent in the tumor-regressed than in mice whose tumor was surgically resected.

Juld also be noted that X5563 tumor-specific im-..nunity. which had been acquired in tumor-regressed mice. was mediated by anti-X5563-TATA-specific Lyt-1⁺2⁻. but not by Lyt-1⁻2⁺ T cells. indicating that the tumor-specific Lyt-1⁺2⁻ T cell population whose generation was augmented through collaboration with TNP-specific helpers primarily exhibited a protective effect (T. Yoshioka. H. Fujiwara. and T. Hamaoka. manuscript in preparation). Because these Lyt-1⁺2⁻ T cells exhibited no cytotoxic effect on X5563 tumor cells in a 4-hr ⁵¹Cr-release assay. further studies are in progress concerning the mechanisms of anti-tumor-specific Lyt-1⁺ T cell function in eradicating tumor cells in vivo.

The most interesting and important finding in the present study (which has not been reported in other tumor-specific immunotherapy experiments) is the demonstration of the applicability of this TNP immunotherapy protocol to an autochthonous tumor system. This finding is worthy of discussion from two perspectives. First. the evidence that TNP immunotherapeutic potential allows the induction of tumor regression to an appreciable proportion in autochthonous as well as transplantable tumor systems clearly emphasizes the validity of this TNP immunotherapy model on the basis of the T-T cell interaction mechanism. This could also provide a theoretical basis for Klein's clinical approaches in which skin malignancies were treated by haptenic reagents (16). Although fu---r investigation is required to explore a chemical t suitable for the in situ modification of human ---rs..the present system may provide a prototye of the immunotherapy of some types of clinical tumors such as skin cancers.

Second. it remains to be proven why 14 of 25 of the ...
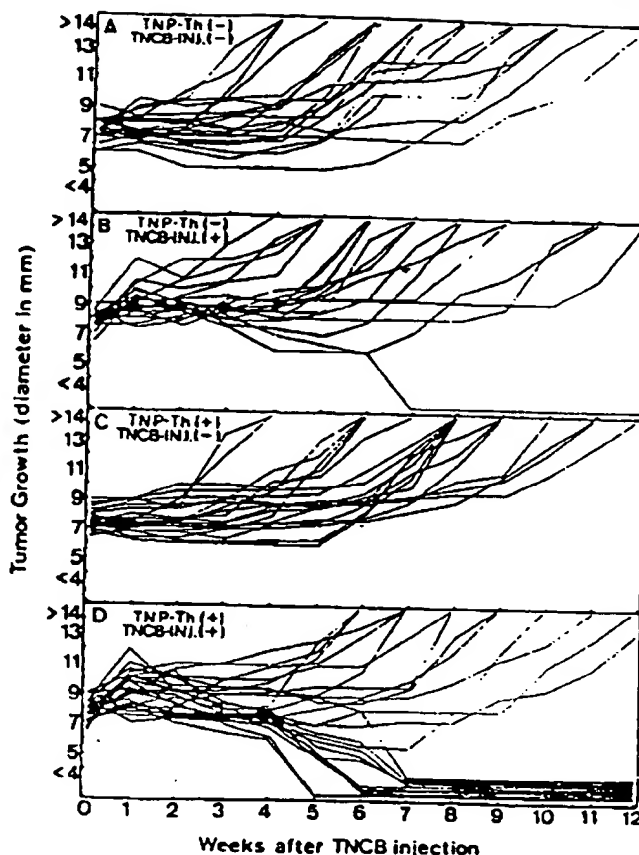

Weeks after TNCB injection

Figure 3. Regression of growing autochthonous tumors by the TNP immunotherapy regimen. C3H/He mice were inoculated subcutaneously with 0.5 mg MCA. Four weeks later. mice received the combined treatment of Cy injection and TNCB painting (groups C and D). Nine weeks after MCA injection. 0.15 ml TNCB in olive oil was administered into subcutaneously growing autochthonous tumors of groups B and D. Group A was MCA inoculation only. Tumor growth was individually scored and expressed by tumor diameter. Tumors in all groups that reached a 14-mm diameter continued to grow for as many as 8 wk ultimately killing the animal. For limitation of the scale. such stage of growth was omitted.

TABLE V

Summary of incidence of tumor regression and mean survival time*

| Group | Treatment | | Incidence of Tumor Regression | Mean Survival Time (weeks ± SE) | No. Dead Mice |
	TNP-Th Induction	TNCB injection			
A	–	–	0/20	13.00 ± 0.75	20
B	–	+	1/20	11.32 ± 0.55	19
C	+	–	0/20	13.60 ± 0.58	20
D	+	+	11/25	12.90 ± 0.68	14

* Determined 20 wk after injection of TNCB into autochthonous tumor and expressed by mean survival time of dead mice at this stage.

been assumed that most of tumors bear TATA (17, 18). the qualitative diversity and quantitative heterogeneity in the expression of each putative TATA on an autochthonous tumor cell has not been well determined. Further experiments are therefore required to determine whether the tumor-specific immunity is in fact acquired in mice whose autochthonous tumor has regressed and how putative TATA in each individual autochthonous tumor qualitatively varies. and to investigate the relationship between the immunogenicity of the autochthonous tumor and the prognosis of the tumor-specific immunotherapy. Such approaches are in progress by challenging the autochthonous tumor cells obtained by excisional biops...